

## The Gametophyte of *Ophioglossum pendulum* in Culture

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**ABSTRACT.**—The spores of *Ophioglossum pendulum* ssp. *falcatum* germinated after six weeks in the dark on a nutrient medium containing inorganic nutrients and glucose. The gametophytes grew on the same nutrient medium to give globular, teardrop-shaped, and finally cylindrical gametophytes. The mature gametophytes were cylindrical and highly branched. Other aspects of these gametophytes were normal for *Ophioglossum* gametophytes with sunken antheridia and short-necked archegonia. The gametangia were functional because fertilization took place in older cultures. Mature gametophytes of *O. pendulum* ssp. *falcatum* from culture had the same structure as those of *O. pendulum* ssp. *pendulum* from nature. The differing conditions under which the gametophytes of both subspecies grew did not alter their stellate form.

**KEY WORDS.**—*Ophioglossum*, gametophytes, fern development

In a study by Whittier and Moyroud (1993), spores of *Ophioglossum palmatum* L. were used to determine if gametophytes of an epiphytic *Ophioglossum* species could grow in culture. The spores germinated but only when the pH was low, which made it difficult to keep an agar-based culture medium solid. Hundreds of small multicellular gametophytes formed, but few advanced beyond the 12-celled stage. About 30 macroscopic gametophytes developed and continued to grow when transferred to new cultures. Only two of these macroscopic gametophytes became mature after two and a half years in culture. Why the nutrient medium on which hundreds of spores germinated did not support the growth of a significant number of older gametophytes is not understood. It was expected with modifications to the nutrient medium that greater numbers of mature gametophytes would develop, however this did not happen.

Because the study on *O. palmatum* gave inconclusive results, this study using spores from the other epiphytic species of the genus, *Ophioglossum pendulum* L., was initiated. The aim of this experiment, as before, was to grow mature gametophytes of an epiphytic species on the solid agar surface of a nutrient medium. Also, it was of interest to determine whether the growth pattern and structure of these gametophytes was the same as those of gametophytes growing under natural conditions.

### MATERIALS AND METHODS

Spores of *Ophioglossum pendulum* L. ssp. *falcatum* (Presl) Clausen were obtained from plants on Mt. Tantalus in Oahu, Hawaii. A voucher is on deposit



at TENN. The spores obtained had the typical shape and internal organization for *Ophioglossum* spores (Whittier, 1981; 2003).

The spores were surface sterilized with 20% Clorox (1.1% sodium hypochlorite) for 2 min by the method of Whittier (1964). Under sterile conditions, the spores were rinsed with water, collected on filter paper, suspended in water and sown on 12 ml of nutrient medium in culture tubes (20 mm × 125 mm) with screw caps that were tightened to reduce moisture loss.

The nutrient medium contained 100 mg  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 40 mg  $\text{CaCl}_2$ , 100 mg  $\text{K}_2\text{HPO}_4$ , and 25 mg arginine per liter. The medium was completed with 2.5 g of glucose, 0.5 ml of a minor element solution (Whittier and Steeves, 1960) and 4 ml of a FeEDTA solution (Sheat *et al.*, 1959). The medium was solidified with 1.1% agar and was at pH 5.7 before autoclaving. The sown spores were maintained in darkness or under a 12 hr photoperiod ( $50 \mu\text{mol} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$ ) from cool white fluorescent lamps at  $22 \pm 1^\circ\text{C}$ . To promote the development of mature gametophytes, young gametophytes were transferred to fresh nutrient medium containing 0.5% glucose instead of 0.25% glucose. Later, mature gametophytes were transferred from the culture tubes to petri plates with 50 ml of nutrient medium containing 0.5% glucose for additional growth.

The mature gametophytes were fixed with Randolph's modified Navashin fluid (CRAF; Johansen, 1940). After fixation, the gametophytes were embedded in paraffin and sectioned by conventional techniques (Johansen, 1940). The sections were stained with Heidenhain's hematoxylin, safranin O, and fast green.

## RESULTS

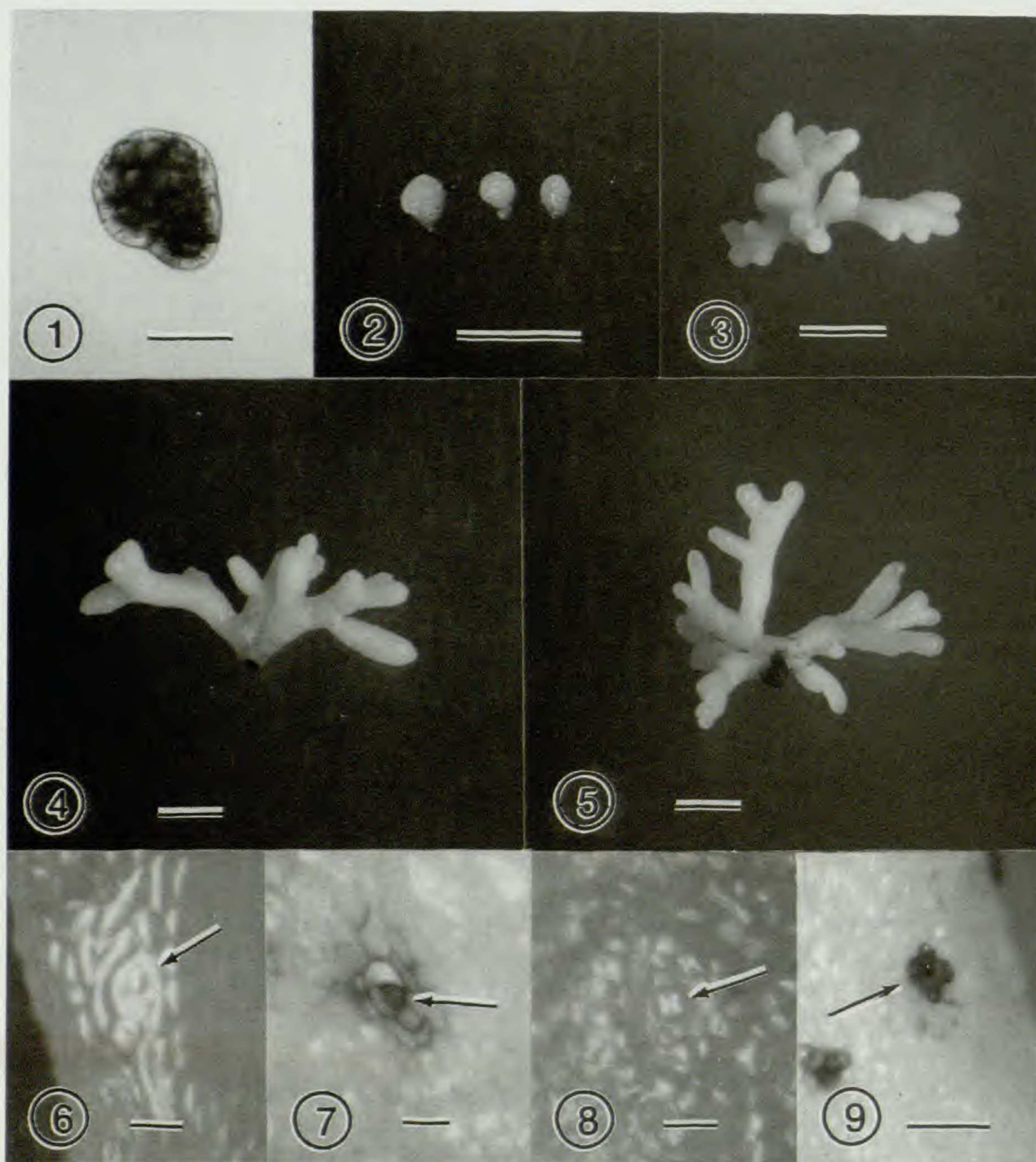
After six weeks (42 days) in the dark, 6.3% of the spores had germinated. Germination must have initiated during the sixth week because germination had not occurred by the end of week five (35 days). No germination occurred in illuminated cultures after one year.

About a month after the first germinating spores were found, small globular gametophytes (Fig. 1) had developed. Six months later, larger teardrop-shaped gametophytes (Fig. 2) were observed on the surface of the nutrient medium.

The teardrop-shaped gametophytes enlarged to initiate short cylindrical gametophytes. At this time the gametophytes began to branch forming cylindrical branches in several directions. These branches had antheridia and archegonia interspersed along their length and thus were sexually mature. Besides increasing in length through the action of meristems each with a single apical cell (not illustrated), the original branches underwent more branching (Figs. 3, 4, 5). Relatively quickly a gametophyte with numerous radiating branches was formed. The branches were brittle and tended to detach if the gametophytes were handled.

The antheridia were difficult to see on the living gametophytes without the use of a dissecting microscope at high magnification (Fig. 6). The exposed portion of the antheridium was in a shallow depression on the surface of the gametophyte (Fig. 6). It was composed of 4–5 cells with a more or less centrally



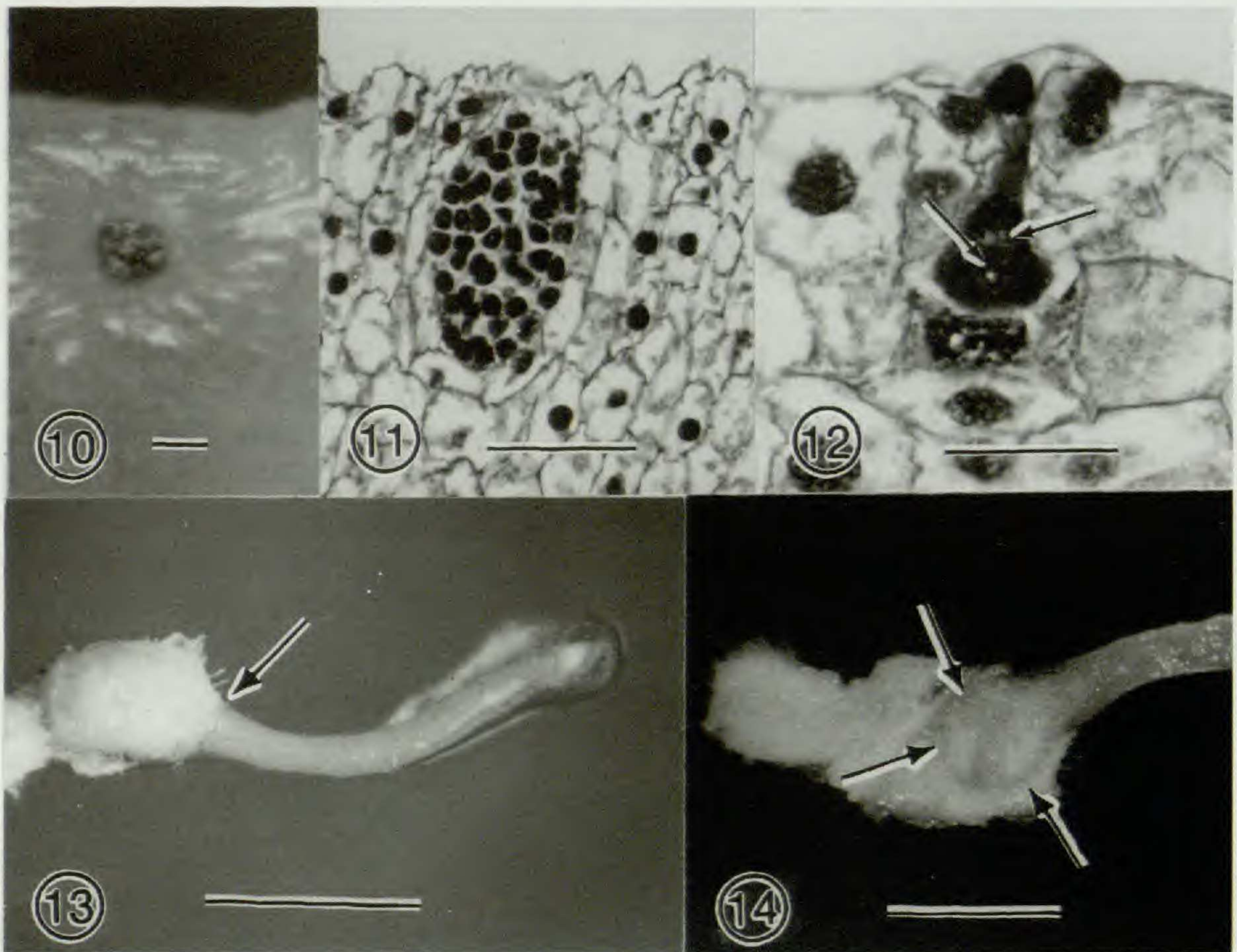


FIGS. 1–9. Gametophytes of *Ophioglossum pendulum*. 1. Globular gametophyte, bar = 100  $\mu$ m. 2. Teardrop-shaped gametophyte, bar = 2 mm. 3–5. Branched gametophytes, bars = 2 mm. 6. Surface view of an antheridium (arrow), bar = 100  $\mu$ m. 7. Surface view of an old antheridium with opercular cell (arrow), bar = 100  $\mu$ m. 8. Surface view of neck of young archegonium (arrow), bar = 200  $\mu$ m. 9. Open archegonium with reflexed neck cells (arrow), bar = 200  $\mu$ m.

located single opercular cell. The triangular surface wall of the opercular cell was best seen with a postmature antheridium having dark walls (Fig. 7).

The archegonia (Fig. 8) were also difficult to find on the surface of these gametophytes. They were best identified with light reflecting off the gametophyte surface. The neck of the archegonium sits in a shallow depression at the gametophyte surface. It had a short neck that extends about 30  $\mu$ m above





FIGS. 10–14. Gametophytes and embryos of *Ophioglossum pendulum*. 10. Open archegonium without reflexed neck cells, bar = 100  $\mu$ m. 11. Longitudinal section of an antheridium, bar = 100  $\mu$ m. 12. Longitudinal section of an archegonium with two nuclei in neck canal cell (arrows), bar = 50  $\mu$ m. 13. Gametophyte with protruding primary root of embryo with arrow showing exit point of root, bar = 2 mm. 14. Handsection of embryo with main mass of embryonic tissue embedded in gametophyte (arrows), bar = 1 mm.

the gametophyte surface. It was composed of a fully exposed terminal tier of four neck cells attached to a partially exposed subterminal tier of neck cells. When an archegonium opened, the neck cells of the terminal tier spread apart to expose the neck canal to the external environment. This was easiest to observe with an old open archegonium that had dark walls (Fig. 9). The reflexed neck cells were easily displaced in handling the gametophyte to show the four neck cells to which the terminal neck cells were attached (Fig. 10).

The antheridia were sunken in the parenchymatous tissue that makes up the body of the cylindrical gametophyte. Close to maturity each antheridium contained an ellipsoidal or spherical mass of spermatocytes (Fig. 11) in longitudinal section. Sections of archegonia clearly demonstrated a short neck, a binucleate neck canal cell and an egg for each archegonium (Fig. 12). A ventral canal cell was not observed. It may have been missed because the correct stage of archegonial development was not sectioned.



Young embryos were formed on gametophytes in old cultures. Enough water existed on the surface of the nutrient medium for the spermatozoa to reach the archegonia and bring about fertilization. The embryos were recognized by the primary root growing out of the gametophyte (Fig. 13). A large portion of the young embryo at this stage remained embedded in the gametophyte (Fig. 14). There was no stem or primary leaf as part of the embryo at this stage. The later development of these young sporophytes was not followed.

#### DISCUSSION

The spores of *O. pendulum* ssp. *falcatum* from Hawaii started germinating in the dark at about the average time for previously studied spores of *Ophioglossum* (Whittier, 2003). Also, their time for germination was close to that reported by Campbell (1907) for spores of ssp. *pendulum* from Sri Lanka and Java. He reported germinated spores 36 days after sowing. In the present study the earliest spore germination needed more than 35 days but less than 42 days in the dark. Because 6% germination and some gametophyte growth had occurred at 42 days, the earliest germination would have been initiated prior to day 42.

The earliest stages of gametophyte development were not examined in this study. However, the young globular gametophytes of *O. pendulum* ssp. *falcatum* (Fig. 1) had the same structure as those of other *Ophioglossum* species (Whittier, 1981; 2003). It appeared from the occurrence of typical globular gametophytes that there was nothing unusual about early gametophyte development in this subspecies.

Highly branched gametophytes of *O. pendulum* ssp. *pendulum* were found by Lang (1902) and Campbell (1907) in Sri Lanka and Java. Both reported that the branches of these gametophytes extended in all directions. Gametophytes of *O. pendulum* ssp. *falcatum* with the same basic structure as those of ssp. *pendulum* were grown in culture. In nature the gametophytes grow embedded in tangles of roots between persistent leaf bases of some ferns or in humus on tree branches, whereas in culture the gametophytes grow on the solid surface of the nutrient medium. Whether the gametophytes grew in nature or in culture did not alter the structure of these highly branched gametophytes.

In addition to the normal growth pattern, the anatomy of these gametophytes was the same as that of gametophytes from nature. The apices had a single apical cell type of meristem and the bulk of the gametophyte was composed of parenchyma tissue. Sunken antheridia with ellipsoidal or spherical masses of spermatocytes were illustrated by Lang (1902) and Campbell (1907). The archegonium with its short neck, binucleate neck canal cell and egg was known from earlier studies. One difference was the apparent absence of the ventral canal cell in the archegonia on the cultured gametophytes of ssp. *falcatum*. Campbell (1907) reported its presence in ssp. *pendulum* but acknowledged that it was difficult to find because it was extremely inconspicuous. Because it forms late in development just before archegonial maturity, it may be evanescent. This along with the small size can explain why



the ventral canal cell was missed in this study and also by Lang (1902). Other than the difficulty in finding the ventral canal cell, the archegonia on the cultured gametophytes of ssp. *falcatum* are the same as those of ssp. *pendulum* from nature.

In support of the normalcy of the cultured gametophytes was the occurrence of sexual reproduction. Several young embryos with the primary root piercing the gametophyte to contact the surface of the nutrient medium occurred on these gametophytes. The embryos consisted of a mass of tissue embedded in the gametophyte and the elongating primary root. No stem or primary leaf was observed at this stage. This structure was described for young embryos of ssp. *pendulum* (Campbell, 1907). Embryos with precocious roots are not unusual for the genus, but where the stem and primary leaf arise on these young embryos is open to question (see Mesler *et al.*, 1975).

This study has demonstrated that the gametophytes of an epiphytic *Ophioglossum* species, *O. pendulum*, can be grown in culture. This study was successful in part because the nutrient medium on which the spores germinated also supported gametophyte development to maturity. The branched gametophytes of ssp. *falcatum* had the same branched structure as those of ssp. *pendulum* from nature. The substrates on which gametophytes of the two subspecies grew had little affect on their basic structure. Stellate gametophytes formed under the growing conditions in nature and those in culture.

#### ACKNOWLEDGMENTS

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